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13. ABSTRACT (Maximum 200 Words) Manipulations capable of repealing host tolerance to induce T cell-mediated prostate tissue-specific responses are of central importance to immunotherapeutic approaches to prostate cancer treatment. Hence in the current proposal, we test whether androgen ablation (by castration) can induce T cell responses targeting murine prostate epithelial and tumor cells. To date, we have found that castration of TRAMP mice results in prostate and tumor infiltration by antigen presenting cells (APC's) as well as CD4+ and CD8+ T cells. These studies complete our original Specific Aim 1 and suggest that castration can prime host responses amenable to immunotherapeutic potentiation. Further studies proposed in our original Specific Aims 2 and 3 will address the potential of androgen ablation to facilitate/potentiate novel immunotherapies, including manipulations that licence APC's or prevent T cell downregulation, for prostate cancer treatment.			
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INTRODUCTION

Our previous studies pertaining to T cell costimulatory-based immunotherapies of murine prostate cancer (1-9), as well as antitumoral immunotherapeutic studies by our collaborators and others (10-14), underscore two principle points: 1) immunotherapies (including CTLA-4 blockade) may prove particularly effective when tumor-induced impairments in host immune function are minimized -- for instance, by diminishing tumor burden and/or reducing the capability of tumor cells to elaborate immune inhibitors; and 2) prior interventions that successfully overcome host tolerance to prime a prostate-specific, cell-mediated immune response should greatly facilitate antitumoral responses bolstered by immunotherapies such as CTLA-4 blockade. Hence, to address these two points within the context of developing potent immunotherapeutic approaches for the treatment of prostate cancer, we originally proposed to test whether androgen ablation might prove useful as a "first step" in converting prostate tumors into their own "*in situ vaccines*" to facilitate a response to immunotherapy. The mechanism(s) we envisioned whereby androgen ablation might induce/facilitate immunotherapeutic responses are encompassed in two non-mutually exclusive paradigms. In the first paradigm, androgen ablation reduces tumor burden thus reducing the number of tumor cells that must be eliminated by an immune response raised by CTLA-4 blockade. Additionally, reduction of tumor burden or downregulation of tumor cell function by androgen ablation may partially alleviate some immuno-suppressive effects of the tumor upon APC and T cell function. In the second paradigm, androgen ablation results in the recruitment of immune cells into tumor sites. Moreover, androgen ablation-induced prostate cell death might result in greater availability tumor-derived antigens that can be appropriate by host APC for presentation to induce antigen-specific activation of T cells whose action can be potentiated by immunotherapies such as CTLA-4 blockade – somewhat analogous to the capability of CTLA-4 blockade to markedly exacerbate pre-existent pathologic inflammatory processes such as murine experimental allergic encephalomyelitis.

BODY

Prompted by the discussion and considerations mentioned above, our current DOD proposal is intended to address our overall hypothesis that: **Androgen ablation combined with CTLA-4 blockade immunotherapy can diminish/prevent the progression of prostate cancer that inevitably occurs following androgen ablative therapy alone.** The following annual report outlines our progress in testing this overall hypothesis by addressing three separate aims we previously outlined:

Specific Aim 1. To Histologically and functionally characterize immune cells that infiltrate prostate tumors following androgen ablation.

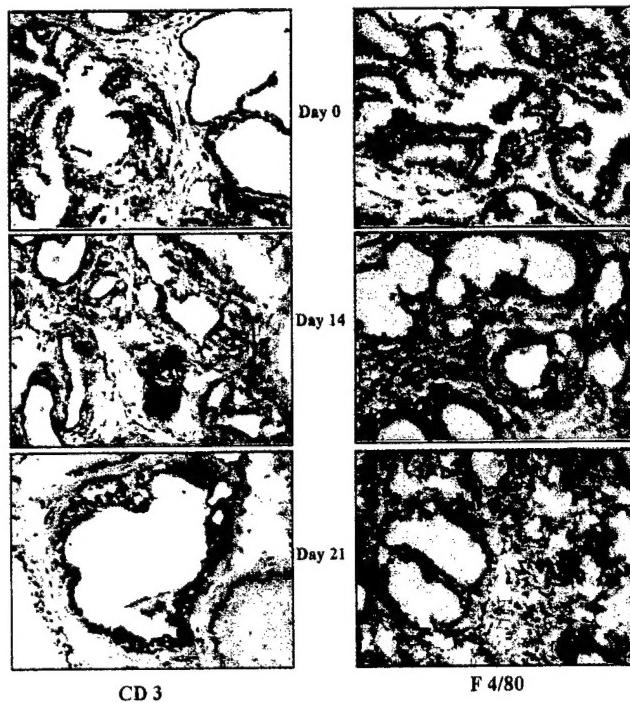
Specific Aim 2. To test the hypothesis that *in vivo* CTLA-4 blockade can diminish the progression of prostate cancer that inevitably occurs following androgen ablative therapy alone.

Specific Aim 3. To test the hypothesis that *in vivo* activation of host antigen presenting cells can potentiate antitumoral responses raised by the combination of androgen ablation and CTLA-4 blockade immunotherapy.

To date, we have nearly completed the objectives of our original **Specific Aim 1**. This includes the following specific tasks outlined for **Specific Aim 1** in our original **Statement of Work**: 1) Breed homozygote TRAMP mice to generate sufficient numbers of breeding-age homozygote animals for heterozygote breeding. Breeding homozygous TRAMP mice for breeding with C57BL/6 mates to generate ≥ 65 heterozygote males

(completed in months 1-5); 2) Surgical castration(or sham castration) of 60 twelve-week old heterozygote males (completed in months 4-5); 3) Harvest and micro-dissection of 60 prostates (ventral, lateral, posterior and anterior lobes) to determine the severity of TRAMP tumor infiltration by B7.1+ and/or B7.2+ APC, and CD8+ T-cells (completed in months 4-7); 4) Immunohistochemical (also H&E) staining of cryosectioned tissues for the phenotypic surface marker analysis of infiltrating cells (CD4, CD8, F4/80 and MIDC-8/NDLC-14) and androgen presenting cell expression of B7.1/B7.2 (completed in months 8-11); 5) Compare number and types of immune cells in castrate versus non-castrate TRAMP males (in progress); 6) Determining time of maximal T-cell infiltration (completed in months 11-12); and 7) Statistical analysis of all data and completion of manuscript (in progress for months 13-16). The following summarizes observations emanating from our experiments outlined in **Specific Aim 1.**

To date, we have established that castration of 12-week old TRAMP mice results in rapid involution of TRAMP prostates containing early prostate tumors. We have also established that the androgen-intact (untreated) TRAMP prostate typically harbors a paucity of mononuclear cells. During prostate involution induced by castration, we have found that a vigorous cellular immune response develops within TRAMP mouse prostates and tumors (comparison of 50 castrated versus 50 sham-castrated mice). TRAMP prostate infiltration by mononuclear cells is readily apparent by 14 days following castration, and increases progressively until at least 28 days following castration. Phenotypic analyses of prostate-infiltrating mononuclear cells, using immunohistochemical staining of cryosectioned tissues (frozen in OCT and sectioned at 5 μ m), demonstrates that potential antigen presenting cells including F4/80+ macrophages (Figure 1, *below*), MIDC-8+/NDLC-14+ dendritic cells (data not shown) and a lesser amount of CD3+ T lymphocytes (Figure 1, *below*) comprise infiltrates that develop within TRAMP prostates following castration.



Effects of Castration on Murine Prostate CD3 and F 4/80 Cell Populations

Legend Figure 1. TRAMP prostates recovered from non-castrate control mice (day 0) and mice castrated for 14 and 21 days were snap-frozen in OCT and cryosectioned at 5 μ m intervals. Subsequent, to screen prostates for the presence of T cells, prostates were indirectly stained using primary anti-CD3 antibody (DAKO) followed by secondary HRP-conjugated antibody to facilitate visualization of phenotype marker-positive cell populations (series of panels: *left*). To screen prostates for the presence of potential antigen presenting cells, prostates were stained using indirect methods involving primary anti-F4/80 antibody (Serotec) followed by secondary anti-X HRP conjugate to facilitate visualization (series of panels: *right*). Figure 1 demonstrates relatively few marker-positive cells in non-castrate (day 0) control TRAMP mice. In contrast, early and progressive infiltration of murine prostates by CD3+T cells and a potential antigen presenting cell population (F4/80+ macrophages)is evident in TRAMP prostates at days 14 and 21 following castration. Figures are representative of ≥ 50 castrates and ≥ 50 control TRAMP mice studied to date.

To further determine whether T cell infiltration in our model occurs consequent to prostate-specific APC-mediated costimulatory T stimulation, we have attempted to assess levels of B7.1 and B7.2 expression by APC's infiltrating the prostate following castration. Attempts to assess B7 expression by immunohistochemistry have been unsuccessful due to non-specific staining of TRAMP prostate tissues using anti-mouse B7.1 and B7.2 antibodies (Serotec). In light of this, we have redirected our approach by employing dual-label immunofluorescence in combination with flow-cytometric analysis to assess B7.1 and/or B7.2 expression by infiltrating CD45+ leukocytes following their recovery from enzymatically-digested TRAMP prostates. Preliminary results from these experiments suggest that percentages of CD45+ cells capable of expressing costimulatory B7.1 and, particularly, B7.2 ligand increase within the prostate following castration (Table 1, *below*).

SUBSET MARKER ANALYSIS OF CD45+ CELLS RECOVERED FROM TRAMP
PROSTATES 14 DAYS AFTER TREATMENT
(DATA ARE % OF MARKER- POSITIVE CD45+ CELLS)

Treatment	% Live Cells, CD45 ⁺	F 4/80	CD80 (B7.1)	CD86 (B7.2)	CD4	CD8
Sham (n=45*)	1.2 ± 0.4	N/A	N/A	N/A	N/A	N/A
Castrated (n= 15)	16.3 ± 6.2	50.0 ± 9.3	14.0 ± 0.7	20.4 ± 9.6	11.0 ± 4.1	9.1 ± 4.7

Legend Table 1. In three separate experiments, 12-14 week old TRAMP mice were either castrated for 14 days or sham-castrated. Subsequently, TRAMP prostates were recovered, minced, and digested by shaking overnight (12 hours) in TRAMP medium supplemented with Dispase at 37°C. Next morning, cell suspensions were passed through sterile nylon mesh and mononuclear cells recovered by Ficoll/hypaque centrifugation. Recovered CD45+ cell populations were then analyzed for specific markers (F 4/80, CD80, CD86, CD4 and CD8) using dual-label direct immunofluorescent staining and flow cytometric analysis. Since CD45+ cells populations were readily measurable within castrated TRAMP prostates, analyses were performed on individual prostates recovered from groups of 5 castrated mice (5 mice used per experiment; experiments repeated 3 times). Since CD45+ cells could not be detected in prostates recovered from androgen-intact (*sham-castrated) TRAMP mice, prostate preparations from three mice were pooled to provide the 5 separate samples within each experiment (15 sham mice used per experiment; experiments repeated 3 times). Despite the pooling of sham prostate preparations, percentages of CD45+ cells remained extremely low precluding the subsequent analysis of phenotypic and surface markers of mononuclear cells recovered from normal TRAMP prostates (denoted by N/A). In contrast, individual prostates for castrated TRAMP mice demonstrated significant percentages of CD45+ cells, with a predominant population of F4/80+ cells, and lesser amounts of CD4+ or CD8+ T cells. Accompanying the increase in a potential antigen presenter (presumably F4/80+ macrophage) is an increase in cells expressing ligands CD80 (B7.1) and CD86 (B7.2) that can serve as competent antigen presenting cells to induce prostate-specific T cell activation via costimulation.

The above data are consistent with our hypothesis that castration induces prostate infiltration by potential competent APC. To further test whether infiltrating macrophages demonstrate potential antitumoral capacity, we have initiated immunohistochemical studies to assess whether castration-induced infiltrating macrophages express inducible nitric oxide synthase (iNOS, data not yet available). In light of recent descriptions of an inverse correlation between numbers of iNOS-expressing APC within prostate tumors and severity of tumor grade, we feel evaluation of iNOS expression by infiltrating APC's may have significant implications for prostate tumor progression following androgen-ablative therapy.

To further elucidate whether prostate T cell infiltration involves a response by CD4+ helper-effector and/or

CD8+ effector T cells, we have recently completed both immunohistochemical (data not shown) and immunofluorescent flow cytometric analysis of T cells within TRAMP prostates following castration (data given above in Table 1). We find that T cells infiltrating the prostate following castration represent an equal response by both CD4+ and CD8+ cells. These data are consistent with a potential for androgen ablative therapy (by castration) to induce prostate tissue-specific T cell mediated immunity. Finally, we are currently in the process of reviewing tissue slides that we previously generated from these experiments to quantify levels of APC and T cell infiltration within the TRAMP prostate following castration.

In anticipation of pursuing our proposed experiments to immunotherapeutically exploit the T cell response that is induced within the TRAMP prostate by castration, we have begun preparing for experiments originally outlined in our **Statement of Work** pertaining to our original **Specific Aims 2 and 3**. Such efforts include: 1) Hybridoma production of 22 mg of anti-CTLA-4 (9H10 cells) monoclonal antibody (completed in months 3-6); Breeding homozygote TRAMP mice with C57BL/6 mates to generate 150 heterozygote males for establishment of treatment cohorts for our initial immunotherapy experiments outlined in **Specific Aim 2** (initiated in months 10-12); and 3) Hybridoma production of 210 mg of AntiCD40 rat monoclonal antibody (10C8 cells) (initiated in months 8-12). The remainder of this year will be directed to the completion of experiments originally outlined in our **Specific Aim 2**

KEY RESEARCH ACCOMPLISHMENTS

- Completed Specific Aim 1. Established that castration in TRAMP mice induces prostate tumor infiltration by mononuclear inflammatory cells.
- Characterized prostate tumor infiltration to establish that castration causes recruitment of potential activated antigen presenters (APC's) as well as CD4+ and CD8+ T cells.
- Established the time-frame for mononuclear cell infiltration within TRAMP prostates that is induced by castration.
- Begun quantification of APC and T cell infiltration that occurs within TRAMP prostates following castration.
- Begun preparations to exploit castration-induced APC and T cell infiltration to potentiate/facilitate immunotherapeutic responses.

REPORTABLE OUTCOMES

A manuscript entitled “Castration-induces infiltration of TRAMP prostate tumors by activated antigen presenters and CD4+/CD8 + T cells” and is currently *in preparation*.

CONCLUSIONS

Observations emanating from our current studies collectively support the hypothesis that castration causes prostate tumor infiltration by both antigen presenting cells and T cells with potential antitumoral effector capabilities. Such a response might serve as a useful “first step” to prompt host prostate tumors (both primary or

metastatic) to act as their own *in situ* vaccines to "jump-start" anti-prostate cancer immune responses. Now that we have characterized this response, including elucidating the kinetics of this response, we feel that prostate tumor infiltration induced by castration might be amenable to selective potentiation by immunotherapeutic manipulation. To fully test this hypothesis, we will continue our studies by testing whether two promising immunotherapies, CTLA-4 blockade and/or anti-CD40 treatment, will act synergistically with castration to induce the complete rejection of prostate tumors in the immunocompetent TRAMP mouse. Our work, to date, has provided the basis for initiating clinical phase I testing of CTLA-4 blockade for the treatment of prostate cancer. It is hoped that findings emanating from our current studies will provide the foundation for clinical phase II studies to test whether androgen ablative therapy can be used to induce/potentiate immunotherapeutic responses in men with advanced prostate cancer.

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APPENDICES: NONE